U.S. Department of Commerce Patent and Trademark Office		Attorney's Docket No. 4817/0R
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. Application No. (if known, see 37 CFR 1.5) 09/530747
INTERNATIONAL APPLICATION NO. PCT/EP98/06961	INTERNATIONAL FILING DATE 3 November 1998	PRIORITY DATES CLAIMED 4 November, 1997; 28 March, 1998; 2 April, 1998
TITLE OF INVENTION SPECIFIC AND SENSITIVE NUCLEIC ACID DETECTION METHOD		
APPLICANT(S) FOR DO/EO/US KESSLER, HABERHAUSEN, BARTL AND ORUM		

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C.371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [X] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [X] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). [unexecuted]
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/530747	PCT/EP98/06961	4817/0R

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):
Search Report has been prepared by the EPO or JPO..... .$840.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
..... .$670.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482)
but international search fee paid to USPTO (37 CFR 1.445(a)(..... .$760.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR1.445(a)(2)) paid to USPTO..... .$970.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... .$96.00

CALCULATIONS/PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
Claims	Number Filed	Number Extra	Rate		
Total claims	9 - 20 =	0	X18.00	$	
Independent claims	1 - 3 =	0	X78.00	$	
Multiple dependent claim(s) (if applicable)			+$260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 840.00	
Reduction by 1/2 for filing small entity, if applicable. Verified Small Entity Statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 840.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 840.00	
			Amount to be refunded :	**$**	
			charged	**$**	

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 500812 in the amount of $ 840.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 500812. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Express Mail No. EK614634676US
Date: May 4, 2000

SIGNATURE

VICTOR K. LEE, Ph.D.
NAME

35,750
REGISTRATION NUMBER

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application
Based on International Application No. PCT/EP98/06961
Filed November 3, 1998
Inventor(s) KESSLER *et al.*

For: **SPECIFIC AND SENSITIVE NUCLEIC ACID DETECTION METHOD**

Attorney Docket No. 4817/0R

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

Alameda, CA 94501
Date: May 4, 2000

Sir:

Prior to examining the above-referenced application as entering the National Stage under 35 U.S.C. §371, please consider the following amendments and remarks.

IN THE CLAIMS:

At page 1, line 1, of the amendment of claims under PCT Article 19, please delete "Claims" and insert therefor --WHAT IS CLAIMED IS: --.

Please amend the claims as follows:

1. (Amended) A method [Method] for the detection of a nucleic acid comprising the steps:

(a) - producing a plurality of amplificates of a section of the [this] nucleic acid with the aid of two primers, one of which can bind to a first binding sequence (A) of one strand of the nucleic acid and the other can bind to a second binding sequence (C') which is essentially complementary to a sequence C which is located in the 3' direction from A and does not overlap A, in the presence of a probe with a binding sequence D which can bind to the third sequence (B) located between the sequences A and C or to the complement (B') thereof, wherein this probe contains a reporter group and a quencher group, using a polymerase having 5' nuclease activity, and

(b) - detecting the nucleic acid by measuring a signal which is caused by the release of the reporter group,

wherein the amplificates formed with the aid of the primers have a length of less than 75 nucleotides.

2. (Amended) The method of [Method as claimed in] claim 1, wherein the binding sequence D of the probe does not overlap one of the binding sequences of the primers.

3. (Amended) The method of claim 1 [Method as claimed in one of the previous claims], wherein at least one of the binding sequences is not specific for the nucleic acid to be detected.

4. (Amended) The method of claim 1 [Method as claimed in one of the previous claims], wherein the total length of the amplificates formed with the aid of the primers have a length of less than 61 nucleotides.

5. (Amended) The method of claim 1 [Method as claimed in one of the previous claims], wherein the probe is labelled with a fluorescence quencher as well as with a fluorescent dye.

6. (Amended) The method of claim 1 [Method as claimed in one of the previous claims], wherein at least one of the primers is not specific for the nucleic acid to be detected.

7. (Amended) The method of [Method as claimed in] claim 6, wherein two of the primers are not specific for the nucleic acid to be detected.

8. (Amended) The method of claim 6 [Method as claimed in one of the claims 6 and 7], wherein the probe is not specific for the nucleic acid to be detected.

9. (Amended) The method of claim 1 [Method as claimed in one of the previous claims], wherein nucleotides which are each complementary to A, G, C and T are used in the amplification.

REMARKS

Applicants have amended the claims to comply with U.S. patent practice in matters of form and to remove certain multiple dependency. After entry of this Amendment, claims 1-9 are pending in this application. The amendments do not introduce new matter. Entry of this Amendment is respectfully requested.

The total filing fee on the Transmittal Letter To The United States Designated/Elected Office (DO/EO/US) Concerning A Filing Under 35 U.S.C. §371 is calculated on the basis of this Amendment.

Respectfully submitted

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Specific and sensitive nucleic acid detection method

The invention concerns a method for detecting nucleic acids in which a section of these nucleic acids is amplified whereby this section must fulfil certain conditions with regard to its base sequence and it also concerns a reagent kit containing two primers and a probe which define this section.

One of the most frequently employed molecular-biological methods for detecting nucleic acids is hybridization with sequence-specific probes to detect homologous nucleic acid sequences. The detection of nucleic acid sequences is important for basic research but is of particular importance in various fields of application e.g. in the fields of medical diagnostics, forensic diagnostics, food diagnostics, environmental diagnostics, plant protection and veterinary medicine.

Either oligonucleotides (short DNA or RNA) or polynucleotides (longer DNA or RNA) are used as probes for this. An advantage of the shorter probes compared to the longer probes is that they have a better sequence selectivity due to the shorter hybridization region but they have the disadvantage of lower sensitivity. An improved sensitivity and sequence selectivity is achieved with PNA probes (peptide nucleic acids, e.g. WO 92/20702) since these probes have a higher binding affinity for nucleic acids (higher Tm) and are characterized by a higher base discrimination (ΔTm). Probes can additionally carry marker groups for nucleic acid detection which are suitable either for capturing and/or detecting hybrid complexes of the probe and nucleic acid to be detected.

In order to detect nucleic acids by hybridization, one or several probes are used either for hybridization in solution or on solid supports. Nucleic acid tests in solution are referred to as homogeneous test formats whereas tests on solid supports and/or mediated by solid supports are referred to as heterogeneous test formats. In the heterogeneous test format the nucleic acid to be detected can be pre-bound to the solid support (e.g. dot blot). Hybridization is carried out by contact with a solution which contains the probe. Conversely, the probe can be pre-bound to the solid support (e.g. reverse dot blot). The hybridization is carried out by contacting the bound probe with a solution which contains the nucleic acid to be detected. Alternatively the complex of nucleic acid to be detected and probe can be firstly formed in solution and subsequently bound to the solid support. In homogeneous test formats probe pairs are for example used which carry terminal energy-transferring groups and are brought into direct contact by co-hybridization to the nucleic acid to be detected and thus generate a signal. Alternatively probes can also be used which, after binding to the nucleic acid to be detected, are converted from a quenched into an unquenched state by means of enzymatic 5' nuclease activity in solution.

The detection of nucleic acids solely by probe hybridization has only a limited sensitivity. Thus only a sensitivity in the pg to fg range is possible even when using sensitive detection marker groups such as ^{32}P, digoxigenin, biotin, fluorescein, ruthenium chelates, fluorescein, rhodamine or AMCA. However, sensitivities in the ag range and a high test specificity is required for a sensitive nucleic acid test especially in the medical-diagnostic field. This

applies to the detection of exogenous nucleic acids e.g. in the form of infectious pathogens as well as to the detection of the presence or absence or change of endogenous nucleic acids. A high test sensitivity and test specificity is, however, also very important in the other stated fields of application.

Thus some infectious pathogens such as e.g. HCV, HIV and HBV have to be detected even when there are only a few copies for a timely medical intervention e.g. by an early drug treatment. The detection of nucleic acid sequences of the pathogen is an advantage for such early tests for pathogens since a sensitive detection is already possible in an early phase of infection (latency phase) due to the availability of nucleic acid amplification methods (nucleic acid multiplication methods). The specific amplification of the agent to be detected is only possible in the case of nucleic acids but not in the case of immunological detection methods. In these methods an increase of the particles that are specific for the pathogen to be detected is only possible by means of the humoral immune response and formation of corresponding antibodies that are specific for the pathogens; however, this immune response only occurs after the latency period and it is a secondary reaction after infection by the pathogen. Therefore detection by means of nucleic acid hybridization has the advantage that the pathogen can be detected very sensitively directly after infection.

However, the success of medical intervention depends not only on being able to detect the pathogen at an early stage with high sensitivity but also very specifically. Therefore in order to treat specifically it is important to differentiate between various pathogens such as e.g.

HAV, HBV, HCV, HIV, various herpes viruses, HPV and to differentiate between individual subtypes such as HIV-1 and HIV-2. In this connection it is also important to have quantitative information and no false-positive or false-negative results since such erroneous results can under certain circumstances have serious therapeutic consequences. This requires accuracy and high reproducibility of the results. Therefore the nucleic acid detection must not only be very sensitive but also very specific and reproducible. The specific and sensitive nucleic acid test must also be carried out rapidly so that specific treatment can begin immediately.

It is often also important to detect several pathogens such as e.g. HCV, HIV and HBV simultaneously e.g. as part of blood bank screening tests. In the current nucleic acid detection tests this is carried out by successive individual determinations of the pathogens to be detected. A disadvantage of this is that several determinations have to be carried out one after the other which is a particular disadvantage when screening a large number of specimens. The availability of sensitive and specific test methods which for example allow a rapid concurrent determination of several pathogens in parallel in a single sample (multiplex determination) is desirable for such nucleic acid determinations.

The availability of specific and sensitive nucleic acid detection methods is also advantageous for the detection of the presence or absence of endogenous nucleic acids within certain genomic loci and/or of changes thereof e.g. hereditary, spontaneous or a mixture of hereditary and spontaneous mutations, deletions, inversions,

translocations, rearrangements or triplet expansions in the form of specific and/or polymorphous changes. However, the availability of specific and sensitive nucleic acid detection methods is not only very important in the medical sector but also in the other fields of applications mentioned above.

The previous test procedures for sensitively and specifically detecting the presence or absence of nucleic acids are based on combined nucleic acid amplification reactions (nucleic acid multiplication) and nucleic acid detection reactions (detection).

For this the nucleic acid to be detected is used in a form that is suitable for the amplification reactions e.g. in the form of untreated or treated sample material and/or sample material concentrates e.g. by adsorption of the untreated or treated sample material to the surface of a solid support and subsequent resorption from this solid support. Such solid supports are for example solid supports with glass-containing surfaces. These solid supports do not substantially purify and/or isolate the nucleic acids to be detected but only result in a concentration of the sample material and may lead to inactivation and/or elimination of inhibitors of the subsequent nucleic acid amplification and detection reactions. These solid supports also enable the provision of several nucleic acids to be detected e.g. in a multiplex method, in a form that is suitable for the nucleic acid amplification and detection reactions.

Other sample preparation methods include specific process steps for the nucleic acid-specific and/or sequence-specific binding of the nucleic acid to be

detected e.g. by using solid supports with nucleic acid-specific binding groups and/or nucleic acid capture probes to selectively bind and release the nucleic acid to be detected by nucleic acid-specific binding and subsequent dissociation between the binding group and/or carrier-bound capture probe and nucleic acid to be detected. Nucleic acid specific binding groups and/or nucleic acid capture probes on the surface of the solid support are necessary for this type of solid support. Thus in order to prepare several nucleic acids to be detected e.g. for a multiplex method, it is either necessary to have several solid supports which is more complicated or to have solid supports with one or several binding groups and/or with multiple or several capture probes. Multiple capture probes contain several binding sequences for several nucleic acids to be detected. These supports with several binding groups and/or several and/or multiple capture probes are, however, more complicated to prepare. In addition it is more difficult to adjust the reaction conditions for the specific binding of several nucleic acids to be detected to a support containing several binding groups or/and capture probes or for binding several types of nucleic acids to be detected to a nucleic acid-specific binding group or to a capture probe with several complementary hybridization sequences.

The amplification and the detection of the prepared nucleic acids to be detected is carried out in heterogeneous or homogeneous nucleic acid amplification test formats. The nucleic acid amplification reactions and detection reactions can either be carried out successively (heterogeneous test methods) or simultaneously (homogeneous test methods). Target-specific nucleic acid amplification reactions, target-

dependent signal-nucleic acid amplification reactions or signal nucleic acid amplification reactions are used as the amplification reactions. Detection systems for detecting amplified nucleic acids are either based on the incorporation of nucleotides and/or the use of labelled primers or labelled probes. The detection systems that are used contain either direct or indirect detection labels or coupled secondary and tertiary detection components. However, the amplified nucleic acids to be detected can also be detected by spectroscopic or physical methods.

The previous nucleic acid amplification detection method with integrated signal-nucleic acid amplification reactions have the disadvantage of lower sensitivity due to the non-exponential signal amplification, increased susceptibility to interference due to a stronger tendency for background signal generation as a result of the large number of probe components and the formation of unspecific detection signals since it is not the nucleic acid to be detected which is amplified target-independently but only a detection signal which is coupled thereto. Examples are coupled signal cascades (e.g. SELF cycle) or signal-generating probe tree or brush structures (e.g. branched DNA).

The previous nucleic acid amplification detection methods with integrated target-dependent signal-nucleic acid amplification reactions are more sensitive than the pure signal-nucleic acid amplification methods due to the exponential increase in signal, but they in turn have the disadvantage that unspecific detection signals are formed since it is not the nucleic acid to be detected as such that is enzymatically amplified in a target sequence-independent manner but only a detection

signal derived in an initial target-dependent primary reaction in the form of a nucleic acid reporter molecule. Examples are the Qβ replication reaction in which a Qβ reporter molecule is amplified enzymatically or the ligase chain reaction in which sections of the nucleic acid reporter molecules are enzymatically linked in a sequence-independent manner.

The nucleic acid amplification products that have been generated by the previously most sensitive and specific exponential target-specific nucleic acid amplification reactions such as e.g. PCR (US-A-4,683,202 or EP-B-0 202 362), RT-PCR, SDA, NASBA (EP-A-0 329 822) or TAM (WO 91/01384) were single or double-stranded nucleic acid amplification products produced by target sequence-dependent thermocyclic or isothermal enzymatic elongation of primers running in opposite directions that are sequence-specific for the nucleic acid to be detected and bind to the ends of the nucleic acid amplification unit (amplicon) of the deoxyribonucleic acids or ribonucleic acids to be detected or to complements thereof and thus restrict the nucleic acid amplification products. All four base specificities are incorporated in these elongation reactions.

The said nucleic acid amplification detection methods with an integrated target-specific nucleic acid amplification reaction are the most specific due to the target sequence-dependent enzymatic nucleic acid amplification cycles. Whereas linear target-specific nucleic acid amplification reactions such as e.g. the cycling probe reaction only lead to a limited sensitivity, exponential target-specific nucleic acid amplification reactions such as elongation-based reactions such as e.g. the polymerase chain reaction

(PCR, RT-PCR, SDA) or transcription-based reactions such as e.g. nucleic acid sequence based amplification (NASBA) or transcription mediated amplification (TMA) have previously resulted in the most sensitive and specific signals.

Although mixed forms of target-dependent signal nucleic acid amplification and target-specific nucleic acid amplification such as e.g. the gap-filling ligase chain reaction (gap-filling LCR, WO 90/01069) have a target-dependent reaction step compared to the non-modified LCR, this is, however, restricted to limited sequence sections that are only composed of 1 or 2 base specificities and thus have a limited target specificity.

Various methods are available to detect the nucleic acid that is formed. Detection of the generated nucleic acid amplification products by means of fragment or sequence gel analysis is time-consuming and non-quantitative. Detection by means of carrier-bound dot, slot or reverse dot blot methods is also time-consuming and non-quantitative.

Sensitive and specific quantitative determinations of the nucleic acids to be detected have previously been possible in heterogeneous or homogeneous target-specific exponential nucleic acid amplification reaction formats in which the nucleic acid amplification product is captured in a part of the sequence section that is formed by elongation either by an incorporated label or by hybridization during or after amplification with a specific probe for the nucleic acid to be detected or its complement. Exponential nucleic acid amplification

reaction formats in which an intercalation of nucleic acid binding dyes occurs are also sensitive but not sequence-specific.

In heterogeneous reaction formats the nucleic acid amplification product is bound to a solid support for example either by means of a primer capture modification or by means of an immobilized capture probe which is complementary to an internal sequence section of the nucleic acid amplification product and is detected as a result of incorporation of a detection-labelled nucleotide, by hybridization with a detection-labelled probe which is complementary to an internal sequence section of the nucleic acid amplification product or by means of a primer detection modification. In homogeneous reaction formats the detection has previously been carried out for example by hybridizing a probe which is complementary to an internal sequence section of the nucleic acid amplification product and which carries a quenched fluorescent label in which case there is a target sequence-dependent enzymatic abolition of the quenching by the primer elongation-dependent release of the quenched fluorescent labelled nucleotide (WO 92/02638) or by the attachment and/or intercalation of a detectable molecule or a group.

Nucleic acid amplification units (amplicons) have been used in all previous quantitative sensitive and specific heterogeneous and homogeneous target-specific exponential nucleic acid amplification reaction formats which have contained additional sequences of variable length between the flanking primer binding sequences and the internal probe binding sequence in addition to the specific primer and probe binding sequences. This five-part amplicon structure resulted in amplicon lengths

that are larger than the sum of the sequence lengths of the two flanking primers and of the internal probe of between preferably 100 and 1000 base (pairs). Optimization of the nucleic acid amplification reaction by improved enzyme mixtures have previously been mainly directed towards longer nucleic acid amplification products.

Shorter amplicon lengths have previously been generated only for the detection of special sequences such as e.g. in triplet expansions, for in-situ examinations or the detection of greatly fragmented nucleic acids as part of age research. However, these short amplicon lengths were detected in time-consuming gel formats or in-situ formats which are characterized by poor sensitivity and/or lack of quantification. Other special short sequences such as short tandem repeats, short interspersed repetitive elements, microsatellite sequences or HLA-specific sequences have previously been only used as primer or probe binding sequences or in combination with other sequences.

The five part nucleic acid amplification products have the disadvantage that, in addition to the specific sequences that bind primers and probe, they have additional sequences which extend the amplicon and reduce the overall specificity with regard to the specificity-generating primer and probe binding reactions.

Previously used longer five-part nucleic acid amplification products have the additional disadvantage of longer primer elongation times and thus longer overall test times. The sensitivity is also limited by

plateau effects of the participating enzymes and substrates which are reached earlier with longer amplicons. A further disadvantage of longer nucleic acid amplification products is an increased competition between the amplicon complementary strand and the detector or capture probe and thus a reduced sensitivity. A further disadvantage is the increasing chance of unspecific binding due to the additional sequences resulting in an increased background and thus lower sensitivity (lower signal-noise ratio). A further disadvantage when the nucleic acid amplification product is bound to carrier-bound capture probes is steric and kinetic hindrance of longer nucleic acid molecules; consequently nucleic acid amplification products of the former lengths have been preferably fragmented before binding to the capture probe. An additional disadvantage is the increased susceptibility to fragmentation within the amplicon sequence and thus destruction of the nucleic acid amplification unit; this leads to a lower reproducibility. An additional disadvantage is that longer nucleic acid amplification products hybridize less specifically at low test temperatures of e.g. 37°C which are preset in conventional nucleic acid analyzers since there is a larger difference to the melting temperature. A further disadvantage of five part nucleic acid amplification products when detecting several different nucleic acid amplification products is that different nucleic acid amplification lengths are formed which make it more difficult to carry out a multiplex test.

The aim of the present invention was to provide an alternative detection method for nucleic acids which has advantages over the previously described methods.

A special object of the invention was to provide a target-dependent exponential nucleic acid amplification method for the highly sensitive, highly specific, reproducible and quantifiable detection of one or several single-stranded or double-stranded nucleic acids which in particular avoids one or several of the said disadvantages.

A further object of the invention was to make the selection of the primer and probe sequences so flexible that it is possible to determine several different nucleic acids to be detected in a standardized reaction format using primer or probe sequences that are preferably partially identical while retaining the overall specificity.

The invention concerns a method for the detection of a nucleic acid comprising the steps producing a plurality of amplificates of a section of this nucleic acid with the aid of two primers, one of which can bind to a first binding sequence (A) of a strand of the nucleic acid and of which the other can bind to a second binding sequence (C') which is essentially complementary to a sequence C which is located in the 3' direction from A and does not overlap A, in the presence a probe having a binding sequence D which can bind to a third sequence (B) located between the sequences A and C or to the complement (B') thereof, wherein this probe contains a reporter group and a quencher group, using a polymerase having 5' nuclease activity and detecting the nucleic acid by measuring a signal which is caused by release of the reporter group characterized in that the amplificates formed with the aid of the primers have a length of less than 100 nucleotides.

The invention also concerns a reagent kit for carrying out this method.

Fig. 1 shows schematically the notation used in the present description for the regions on the nucleic acid to be detected.

Fig. 2 shows the corresponding notation for the elongation products of the primers formed as intermediates as well as for the amplificates (amplicons). It also shows that the amplificates can have one or several additional regions Y which are outside the region that contains the sequence information derived from the nucleic acid to be detected.

Fig. 3 shows schematically the arrangement of the binding sequences of the primers and probe in the present invention. There are various alternatives I to VI depending on whether and how the binding sequences overlap. Only one strand of the amplificate is shown in each case. The same arrangement (only complementary) can be constructed for a second strand of the amplificate. The picture is similar for the elongation products formed as intermediates. Cases V and VI show that, in addition to the binding sequence D, the probe contains additional regions X which can be the same or different and do not form base pairs with the amplificate. The prior art case is shown as VII for comparison; the sequences Z represent the additional sequences of the five part amplicon.

Fig. 4 shows a particularly suitable region of the HCV genome for performing the method according to the invention and a sequence from which the primer and probe sequences are preferably selected. This second sequence

is taken from the non-human pathogenic virus HGBV-B. The selected primer and probe sequences are therefore sequences that are not specific for HCV (M. Med. Virol. 48, 60-67).

Fig. 5 shows further preferred primers and probes.

Fig. 6 and 7 show additional primers and probes.

Fig. 8 shows the particularly preferred HCV region which should completely or partially be provided as a template for the amplification. It should not contain adjoining sequences.

Nucleic acids which can be detected with the method according to the invention can be of any origin such as nucleic acids of viroidal, viral, bacterial or cellular origin or from yeasts or fungi. Samples which contain the nucleic acid sequences to be detected or complements thereof are for example human, animal, bacterial or plant liquids or liquids from yeasts or fungi, excrements, smears, cell suspensions, cultures or tissue, cell or liquid biopsies. The nucleic acids are preferably present in solution. In order to realize the full advantages of the method according to the invention it has proven to be advantageous when the nucleic acid to be detected has a size of at least 40 bp. However, the nucleic acid can also be a nucleic acid prepared by cloning, amplification, or in vitro or in vivo replication.

The nucleic acid to be detected can be single-stranded (especially in the case of RNA) or completely or partially double-stranded (especially in the case of

DNA). In the case of double-stranded nucleic acids, both strands can be amplified or only one of them. Single or double-stranded amplificates can be formed from both types of nucleic acids and one or both can be used for the subsequent detection. The sequence of the probe or the probes is selected accordingly. It is preferably complementary to the strand of the amplificate which is used for further detection.

Positive or negative control nucleic acids or quantification standards which have been treated identically to the nucleic acids (internal or external standard, internal or external control) to be detected can be added to the sample or to a control sample. Suitable standards are for example internal or external, heterologous or homologous DNA or RNA standards containing probe binding sequences that are homologous to primer binding sequences or are heterologous to the sequences of the nucleic acids to be detected. Conversely it is also possible to use primer binding sequences that are heterologous especially in the 3' priming region and homologous probe binding sequences. Analogous specimens are preferably used as negative controls which do not contain the nucleic acids to be detected or complements thereof.

The sample is preferably subjected to one or several pretreatment steps before amplification in order to convert the nucleic acids to be detected into a form which can be amplified. In a first optional step the sample (specimen) is pretreated such that the sample is brought into a form from which the nucleic acid to be detected can be converted into a form suitable for converting the pretreated sample into a suitable form for amplification (e.g. separation of interfering

components from the sample).

The type of sample pretreatment depends on the sample type and the complexity of the biological material in the sample. In the case of human body fluids such as e.g. human blood, blood cells are firstly separated in a preferred embodiment in order to produce plasma, serum or blood cell concentrates. This separation step and sample pretreatment considerably reduces the complexity of the biological sample material in the resulting fractions without substantially isolating the nucleic acid to be detected. In the case of sputum or smears the sample is pretreated for example by suspending the sputum or the smear in a liquid or in the case of urine for example by centrifuging and processing the fractions that are obtained. In the case of tissue biopsies the specimens are pretreated for example by suspension and treatment with an agent that dissolves the cell formations. Samples of cerebrospinal fluid are pretreated for example by centrifugation and processing the fractions obtained. In these cases the sample pretreatment also reduces the complexity of the biological sample material.

This can be followed by a step in which the nucleic acid to be detected from the pretreated sample is converted into a form that is suitable for amplification. Known methods are preferably used for this. In a preferred embodiment the pretreated sample is lysed in a first reaction step to release the nucleic acid to be detected e.g. by proteinase K treatment at elevated temperatures or by alkali in the case of deoxyribonucleic acids. In a second step the sample pretreated by lysis is concentrated by attachment to the surface of a solid support and subsequent resorption from this solid

support after addition of chaotropic agents such as e.g. guanidinium hydrochloride or urea in the presence or absence of soluble alcohols such as e.g. isopropanol. Such solid supports are for example solid supports with glass-containing surfaces (e.g. magnetic particles, glass fleeces with glass-containing surfaces, particles, microtitre plates, reaction vessels, dip-sticks or miniaturized reaction chambers which can in turn also be a part of integrated reaction chips). These solid supports preferably result in a non-sequence specific purification i.e. there is not a substantial isolation of the nucleic acids to be detected from other nucleic acids but only a concentration of sample material (nucleic acids) and optionally an inactivation and/or elimination of inhibitors of the subsequent nucleic acid amplification and detection reactions. These solid supports also enable several nucleic acids to be provided in a form that is suitable for nucleic acid amplification and detection reactions e.g. as part of a multiplex method.

In another embodiment the nucleic acid to be detected from the pretreated sample can be converted after nucleic acid release in a first step by for example proteinase K treatment at elevated temperatures or by alkali in the case of deoxyribonucleic acids. In a second step the lysed pretreated sample is contacted with solid supports which are specifically modified with nucleic acid-specific binding groups and/or capture probes in order to selectively bind the nucleic acid to be detected and subsequently the bound nucleic acid to be detected is eluted again by dissociation between the binding group and/or carrier-bound capture probe and nucleic acid to be detected. Examples of nucleic acid-specific binding groups are PNA homopyrimidine oligomers

such as e.g. $(T)_7$-PNA or nucleic acid-binding low molecular substances such as e.g. nucleic acid intercalators, major groove-binders or minor groove-binders. Examples of capture probes that are specific for the nucleic acid to be detected are nucleic acid oligomers or nucleic acid polymers that have binding sequences for one or several nucleic acids to be detected. Other examples of capture probes that are specific for the nucleic acid to be detected are PNA oligomers that have binding sequences for one or several nucleic acids to be detected. The nucleic acid-specific binding groups or the capture probes can be bound to the solid support with or without intermediate spacers either covalently or by means of binding pairs such as e.g. biotin:streptavidin or Ni:chelate.

The nucleic acid sequences used for amplification can be linear or circular and contain sequence modifications and/or other modifications such as e.g. natural or artificial nucleotide analogues or equivalents thereof or base analogues or equivalents thereof or can be methylated, capped, polyadenylated or modified by other means. The nucleic acids or complements thereof used for the amplification can be of natural origin, or they can be fragmented, modified or enzymatically, e.g. with the enzyme uracil deglycosylase (UNG), or physically pretreated, preamplified, or be produced chemically, photochemically or enzymatically e.g. by chemical oligonucleotide synthesis or in vitro replication, in vitro reverse transcription or in vitro transcription.

In the first essential step of the method according to the invention a segment of the nucleic acid to be detected is amplified. This segment is also referred to as an amplicon in the following. It is essential that

this contains the sequence region between the outer ends of the binding sequences A and C' or of the complement thereof of the primers (the primer binding regions) and contains the binding region E of the probe or of the complement thereof. According to the present invention the amplicon (preferably the total length of the sequences of the regions A, B and C) is preferably shorter than 100 nucleotides, particularly preferably shorter than 60 nucleotides, but preferably longer than 40 nucleotides. However, this does not mean that the total length of the amplificates cannot be larger e.g. when the primers have additional nucleotides that do not lie within the binding regions. Amplification methods are used which allow an amplification of the nucleic acid to be detected or the complement thereof and result in the formation of tripartite mini-nucleic acid amplification products [mini chain reaction (MCR)]. In principle all nucleic acid amplification methods that are known in the prior art can be used for this. Target-specific nucleic acid amplification reactions are preferably used. Theoretically exponentional target-specific nucleic acid amplification reactions are particularly preferably used in which an anti-parallel replication of the nucleic acid to be detected or of its complement is carried out e.g. elongation-based reactions such as the polymerase chain reaction (PCR for deoxyribonucleic acids, RT-PCR for ribonucleic acids). Thermocyclic exponential elongation-based nucleic acid amplification reactions are particularly preferred such as e.g. the polymerase chain reaction. The nucleic acids to be detected or complements thereof which are used for the amplification can be present in the form of single-stranded or double-stranded deoxyribonucleic acids or ribonucleic acids. The aim of the amplification reactions is to produce numerous amplificates of a

segment of the nucleic acid to be detected. Hence an amplificate is understood as any molecular species produced by using sequence information of the nucleic acid. In particular the term refers to nucleic acids. The term "amplificate" includes single-stranded as well as double-stranded nucleic acids. In addition to the regions containing the sequence information of the underlying nucleic acid (amplicon), an amplificate can also contain additional regions which are not directly related to sequences of the nucleic acid to be amplified that are outside the ends of the primer binding sites which face away from another. Such sequences with a length of more than 15 nucleotides preferably do not occur on the nucleic acid to be detected or its complement and cannot hybridize with it by direct base pairing. Hence amplificates can either hybridize with the nucleic acid to be detected itself or with its complement. Amplificates are for example also products of an asymmetric amplification i.e. an amplification in which the two strands are formed in different amounts (e.g. by using different amounts of primers) or in which one of the two strands is subsequently destroyed (e.g. by RNase).

A primer in the sense of the present invention is understood as a molecule which can bind by means of base pairing to a nucleic acid T or its complement and which can be elongated preferably enzymatically. Oligonucleotides are preferred which can be elongated at their 3' end using the nucleic acid to be detected or a complement thereof as the template nucleic acid. Monovalent or multivalent or monofunctional or multifunctional agents can be used as primers which allow a nucleic acid-dependent elongation. These agents can also be composed of various types of molecules e.g.

chimeras of PNA and nucleotide(s) or of protein/peptide and nucleotide(s). Preferred primers are oligomers or polymers with a binding length between 9 and 30 nt, especially preferably between 11 and 22 nt which bind anti-parallel to the nucleic acid T to be detected or its complement and which act as one of several reaction partners for an enzymatic replication of the nucleic acid to be detected or its complement. Oligomers are particularly preferably used as primers which, after adding an amplification regent, initiate a directed replication of one or both strands of the nucleic acid to be detected or of its complement by attachment of at least a part of the primer to the nucleic acid to be detected or to its complement. An example of a particularly preferred primer is a deoxyribooligo-nucleotide with a free 3' hydroxyl end.

The agents used as primers can in principle contain additional groups such as labels. The primers preferably do not contain modifications which would later be used to detect or immobilize the amplificates. It is necessary that the primers have the required binding properties to the nucleic acid to be detected or its complement and can be elongated.

A probe is understood as a molecule which can hybridize with nucleic acids as a result of base-base interactions and which can be partially degraded with the aid of the 5' nuclease activity of Taq polymerase, Tth polymerase or other polymerases having 5' nuclease activity or with cloned, mutated or chimeric polymerases having 5' nuclease activity. Hence preferred probes are oligodeoxy-ribonucleotides. The length of a probe with reference to the binding sequence D is preferably between 9 and 30 bases. The probe has preferably a reporter group on

various nucleotides that can absorb light of a wavelength and a quencher which can completely or partially take up the energy that was irradiated and absorbed by the reporter group so that emission of light is quenched by the reporter group. WO 92/02638, US-A-5,210,015 and EP-A-0 699 768 describe details for producing such a probe as well as general conditions for carrying out such a detection method based on this principle. Explicit reference to the disclosure of these patent applications and their equivalents is made herewith.

A binding sequence is preferably understood as the sequence of bases located between the outermost bases of a particular nucleic acid, primer or probe which bind to a particular nucleic acid, primer or probe via base-base interactions including these outermost bases.

The agents used as a probe can contain one or several binding sequences D for one or several nucleic acids to be detected or their complements and especially for one strand of the amplificate and can contain sequence modifications, terminal and/or internal sequence extensions and/or other modifications such as e.g. natural or artificial nucleotide analogues or equivalents thereof, non-functional nucleotide analogues or equivalents thereof or base analogues or equivalents thereof or they can be methylated, capped or poly-adenylated or be modified in other ways provided binding to one strand of the amplificate and degradation by a 5' nuclease is possible. The reporter group preferably lies in the 5' direction from the quencher group at a distance which still allows an effective quenching.

In the present invention the segment of the nucleic acid from which it is intended to produce a plurality of amplificates is selected such that it contains three regions A, B and C. Regions A and C are regions selected such that one primer can use sequence A as the binding sequence and the complement of the region C can serve as the binding sequence for the other primer. A complement within the sense of the present invention is understood as a nucleic acid or nucleic acid sequence which is essentially complementary to a certain other nucleic acid e.g. a sequence region e.g. of an amplificate or of the nucleic acid to be detected.

Essentially complementary means that the base pairs are selected such that (in the case of a hybridization with another nucleic acid e.g. a probe or a primer) a hybridization can still occur under the test conditions or (in the case of an extension product of a primer relative to the template used) that it is possible to form the nucleic acid by a primer extension reaction using the corresponding nucleic acid. Hence essentially complementary often means that more than 90 % of the bases of the nucleic acid or sequence in question can form base pairs with the certain nucleic acid or sequence under stringent conditions.

Regions A and C are preferably of sufficient length according to the invention that conditions can be found under which primers of a corresponding length can hybridize with bases in these regions. Hence the regions are preferably longer than 8, particularly preferably longer than 12 nucleotides. There are also preferred ranges in the sense of the invention with regard to the upper limit of the length of the regions A and C. Regions A and C are each preferably shorter than 30 and

particularly preferably shorter than 20 nucleotides. In a special aspect of the invention the upper length of the regions is limited by the fact that the primers should be able to hybridize to them in an unspecific manner for the nucleic acid to be detected. Hence the particularly preferred length of the binding sequences A and C is 12 to 20 nucleotides. The regions A and C do not overlap on the nucleic acid to be detected.

For the purposes of the invention the segment of the nucleic acid to be detected (which corresponds to the amplicon) and hence the amplificates that are formed from this contain a sequence B that is located between the regions A and C (fig. 1 to 3). This sequence has a length of one or several nucleotides, preferably more than 4, particularly preferably more than 8 nucleotides. The maximum length of sequence B is limited by the required total length of the amplificate. In a preferred embodiment sequence B does not contain nucleotides which do not belong to the binding sequence of the probe. Hence sequence B is preferably smaller than 30, particularly smaller than 15 nucleotides. Sequence B preferably has a length of between 4 and 30 nucleotides. The length of sequence B is particularly preferably between 8 and 15 nucleotides. This sequence or the complement thereof also serves to bind the probe for the purposes of the invention. The length of the probe is selected such that a hybridization with the amplificate is possible. The sequence of the probe is selected such that it contains a binding sequence D which is defined by the nucleotides of the probe which form base-base interactions with the amplicon and especially the nucleotides of the probe that have a base interaction with the outermost corresponding bases of the amplicon. The probe is essentially complementary to the

nucleotides of the binding sequence E of the amplificate. The binding sequence D or D' can be 100 % complementary to the amplificate but also have mismatches between the outer ends of the binding sequence. In addition to the binding sequence, the probe can contain additional groups or residues or nucleic acid binding regions (fig. 3, V, VI).

Various cases can be constructed depending on the length of the region B and the length of the binding sequence D or D'. In a first case the binding sequence D or D' is longer than the region B or B' of the amplicon. In this case the binding sequence D or D' extends into one or both regions A or A' and C or C' of the amplicon. These cases are shown in fig. 3, II to IV. In these cases the amplificate contains no nucleotides between the ends of the regions A and C that face away from one another which do not belong to the binding sequence E or to the binding sequences of the primers. In fig. 3, II and III the binding sequence D of the probe overlaps with one of the two binding sequences of the primers.

In a further case the length of the region B corresponds to the length of the region D such that the binding sequence of the probe does not overlap with the binding sequences of the primers (fig. 3, I). Within the sense of the invention region D or D' preferably does not overlap region A, A', C or C' located in the 5' direction.

In a preferred embodiment the method according to the invention comprises the formation of three-part mini-amplicon (tripartite mini-amplicon) which apart from the sequences binding the primers and probe, have no

additional sequences and thus avoid the disadvantages of forming longer nucleic acid amplification products while, on the other hand, the specificity of the overall amplification format is ensured by the binding of the primers, by the binding of the probe and by the course of the target-dependent enzymatic elongation reaction with all 4 nucleotide or base-specificities or natural or artificial analogues, isomers or equivalents thereof. The amplification method according to the invention is therefore also referred to as a mini-chain reaction (MCR).

If not stated otherwise in the following, the amplification of the nucleic acid sequences to be detected or their complements is carried out using the reaction steps and reaction conditions known to a person skilled in the art. One difference to conventional methods is the use of the specially selected primers and probe sequences which allow the formation and amplification of the tripartite mini-amplicons. An essential feature of the invention is the addition of one or several primers which bind to the primer binding sequences of the nucleic acid to be detected, of the tripartite mini-amplicon or to their complements.

It is common to add amplification reagents that enable amplification. Enzymatically active components (e.g. enzymes) in combination with elongation substrates and suitable auxiliary reagents (such as buffers) can be preferably used as amplification reagents. Preferred elongation substrates are nucleic acid building blocks or natural or artificial analogues or isomers or equivalents thereof. Agents are used as elongation substrates that are suitable for constructing the complementary strand of the nucleic acid to be detected.

Nucleotides are preferably used as elongation substrates. Preferred nucleotides are dATP, dGTP, dCTP, dTTP and/or dUTP, dITP, iso-dGTP, iso-dCTP, deaza-dGTP and ATP, GTP, CTP, UTP and/or ITP, deazaGTP, iso-GTP, iso-CTP.

In the case of PCR the particularly preferred nucleic acid amplification reagents are mixtures of metastable or thermostable enzymatic DNA polymerase activities and mixtures of deoxyribonucleotides and/or ribonucleotides and suitable auxiliary reagents e.g. Taq-DNA polymerase in combination with dATP, dGTP, dCTP, dTTP and/or dUTP and auxiliary reagents such as e.g. salts and optionally detergents. Amplification reagents that are particularly preferably used in the case of RT-PCR are mixtures, complexes or domains of thermostable enzymatic reverse transcriptase and DNA polymerase activities and mixtures of deoxyribonucleotides and ribonucleotides and suitable auxiliary reagents e.g. mixtures of AMV or Mo-MLV reverse transcriptase.

2-phase or 3-phase cycles and preferably 2-phase cycles are carried out for the thermocyclic amplification reactions (e.g. PCR, RT-PCR). In the 2-phase cycles the strand separation of the nucleic acid amplification products is carried out at a high temperature, preferably at 85°C - 95°C, the common primer and probe annealing and primer elongation is carried out at temperatures near to the melting point between the primer and elongation opposite strand or primer and probe and elongation opposite strand, preferably between 48°C and 72°C. The strand separation is carried out by supplying energy and/or enzymatically, preferably by an elevated temperature, microwaves or applying a potential via a microelectrode, particularly preferably by means

of an elevated temperature. Up to 80 thermocycles are carried out and preferably up to 60 cycles. The incubation is carried out for up to 4 hours and preferably for 30 - 120 minutes. The amplification reaction can be carried out in reaction vessels, capillaries or miniaturized reaction chambers which can also be part of an integrated reaction chip. The fluorescence is measured continuously or within short time intervals during the amplification reaction, preferably several times during a temperature cycle, particularly preferably 3-times or more often during a temperature cycle. The amplification reaction can contain an internal control and/or calibrator. The control can also be carried out externally by an additional amplification reaction.

When dUTP is used instead of or in addition to dTTP, dUMP instead of dTMP is incorporated by the DNA polymerase activity into the amplified nucleic acid sequence or its complement. This allows fragmentation of the amplification product and thus of its property as a nucleic acid amplification unit by incubation with the enzyme activity uracil deglycosylase, preferably with a thermolabile form of the enzyme activity in which the renaturation of the enzyme activity occurs more slowly after thermal denaturation. The UMP-containing amplification product can be incubated after the nucleic acid amplification and detection reaction (sterilization) and/or before a new nucleic acid amplification reaction (carry over prevention).

Psoralens and/or isopsoralens and derivatives thereof plus irradiation with UV light can be used alternatively to functionally inactivate the nucleic acid amplification product.

The formation of the amplificates is detected with the probe which binds to the binding sequence B of the amplicon to form a hybrid. It acts as substrate to release a reporter group therefrom. The ends of the binding sequence of the probe are between the outer ends of the primer binding sequences. The probe can thus hybridize with one strand of the amplificate.

Known conditions can be utilized for the probe binding since the method according to the invention is a special embodiment of the so-called hybridization tests which are known in outline to a person skilled in the field of nucleic acid diagnostics. Should experimental details not be elaborated in the following, complete reference is made to "Nucleic acid hybridization", editor B.D. Hames and S.J. Higgins, IRL Press, 1986, e.g. in chapters 1 (hybridization strategy), 3 (quantitative analysis of solution hybridization) and 4 (quantitative filter hybridization), Current Protocols in Molecular Biology, Ed. F.M. Ausubel et al., J. Wiley and Son, 1987 and Molecular Cloning, Ed. J. Sambrook et al., CSH, 1989. The known methods also include the chemical synthesis of modified and unmodified oligonucleotides and the selection of hybridization conditions which can achieve a specificity which, among others depends on the extent of homology between the nucleic acids to be hybridized, their GC content and their length.

In the method according to the invention the probe is either added before or during the amplification reaction preferably in the form of a solution and optionally together with the primers. The reagent conditions are adjusted to allow hybridization of the probe with an amplificate.

Binding between the amplified nucleic acid sequence of the amplicon and/or its complement and the probe takes place during the amplification so that the enzyme can degrade the probe from its 5' end during elongation of the primer. This releases the reporter group preferably in the form of mononucleoside building blocks. Consequently the quenching process is stopped and the reporter group can emit light which is measured as a signal and used to indicate the presence of the nucleic acid.

If several probes or multifunctional probes or probes which have several binding sequences for amplificates of various nucleic acids to be detected or their complements are used, it is possible to bind several different amplificates or complements thereof. In this case the formation of tripartite mini-amplicons preferably of a similar length and particularly preferably the formation of tripartite mini-amplicons of the same length allows uniform incubation conditions to be set in the nucleic acid amplification for the formation of the various binding complexes. This allows a concurrent and/or sequential detection of several nucleic acid sequences in a multiplex method. A multiplex amplification method is usually understood as a method in which either different sequences on a nucleic acid (e.g. different regions of a gene) or different sequences on different nucleic acids e.g. from different organisms e.g. different viruses are amplified simultaneously in one amplification mixture. Such methods make high demands on the reaction conditions since the amplifications for the various sequences must have a similar amplification efficiency for a reliable analysis. It is a subject matter of the present invention to exclude one of the factors causing

differences in efficiency. For this purpose the amplicon lengths preferably do not differ by more than 20 % and particularly preferably by not more than 5 nucleotides.

In a special embodiment of the multiplex method according to the invention, amplicons for the various sequences are prepared and subsequently the sum of the amplicons that are formed is determined. A detection method is preferably used for this in which one label can be used for all detections; thus for example all probes for the individual amplificates can be labelled identically e.g. with the same ruthenium complex. This procedure is particularly advantageous for testing samples from blood banks since it is not the type of infection which determines the suitability of the samples for blood donations, but rather the sample is already disqualified as blood donor material if any tested infection (e.g. HIV or HBV) is present.

In a multiplex amplification method one differentiates between genuine and non-genuine multiplex methods. In the case of non-genuine methods the primers are selected from strongly conserved regions of the analyte nucleic acids such that all nucleic acid sequences to be detected are amplified with one set of (2) primers. In genuine multiplex methods a mixture of more than 2 primers is used, of which at least 2 have a different selectivity. One or several of the primers can be specific for all or for a subset of the nucleic acids to be detected. This method is especially preferred when it is intended to concurrently amplify less related sequences.

Diverse combinations of nucleic acid sequences to be

detected can be amplified concurrently by multiplex methods e.g. different subtypes of a virus or bacteria of various genera or species.

The released reporter group can be detected by methods known to a person skilled in the art, in particular in various embodiments, preferably based on fluorescence measurements.

In the case of quenched fluorescent labels, fluorescence is activated by dequenching after the detection probe binds to the tripartite mini-amplicon that is formed and 5' nucleolytic degradation and release of the nucleotide modified by the fluorescent dye. The resulting fluorescence signals are preferably measured by real time measurements. In a preferred embodiment fluorescein and rhodamine or derivatives thereof are used in the case of the quenched detector probes as fluorescent and quencher components. In a further embodiment ruthenium or rhenium chelates and quinones or derivatives thereof are used as electrochemiluminescent and quencher components in the quenched detector probes.

Embodiments are particularly preferred in the sense of this first aspect of the invention in which at least one of the binding sequences of the primers and the probe is not specific for the nucleic acid to be detected. A sequence is specific in the sense of the invention when, as a result of a consecutive sequence of nucleobases, it would in principle be able to bind under stringent conditions only to one sequence on the nucleic acid to be detected but not to nucleic acids of other organisms or species or groups of organisms that are not to be detected. A sequence is preferably not specific for a

sequence when it hybridizes with other nucleic acids under the conditions that are used to carry out the test.

Independent of the previously described first aspect of the invention, an overriding subject matter of the invention is a method for the specific detection of a nucleic acid comprising the steps of producing a plurality of amplificates of a segment of this nucleic acid with the aid of at least two primers, contacting the amplificates with a probe which can bind to the amplificate and detecting a hybrid formed from the strand of the amplificate and the probe, wherein at least one of the primers is not specific for the nucleic acid to be detected. In this case the region B can contain nucleotides which do not belong to the binding sequence E. However, in this case it is also possible that the binding sequences of the primer and the probe overlap.

Homologies to other genomes (sequences) can be identified with the aid of a defined initial sequence. A search engine with the name "BLAST" (basis local alignment search tool) that is accessible to anyone via the internet (homepage address:>http://www.ncbi.nlm.nih.gov/BLAST/<) can for example be used.

This enables access to diverse other sequence and protein data banks, the most important of which are:

genBank, EMBL, DDJB, PDB, PIR and Swiss-Prot.

BLASTN methods according to Altschul et al. (1990) J. Mol. Biol. 215: 403-410 using the UWGCG search method

are also used.

The search procedures are also used for sequence data banks such as e.g. the EMBL sequence data banks and preferably also viral sequence data banks such as e.g. em-vrl.

The Blast program offers the user numerous adaptations to enable an individual search to be carried out i.e. to identify those sequences which are specific for one or several analytes or which are not specific i.e. which also occur in other organisms or not. In this connection reference is also made to Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, David J. Lipman (1990). Basic local alignment search tool, J. Mol. Biol. 403-410. Surprisingly the selectivity of the detection method is not solely derived from the selectivity of the individual primers for a specific target but rather from the cumulated selectivity of the overall system. Thus two primers or two primers and a probe may even be individually completely unselective i.e. hybridize individually with numerous targets. However, since the selectivities of the individual primers and probes are (only) superimposed in the nucleic acid to be detected this results in an overall specificity. However, since the selectivity of the primers is not so firmly fixed when selecting the nucleic acid to be amplified and detected, it is much easier to localize short amplicons for different targets whose lengths completely or substantially (i.e. more than 95 %) agree. This makes simultaneous amplifications and hybridizations (such as in the case of nucleic acid probe arrays) easier to achieve and reproduce.

The invention also concerns a reagent kit for carrying out this method. This contains the primers and preferably also a detection probe. However, it can also contain additional reagents such as buffers and enzymes e.g. a polymerase.

The primers preferably bind to the binding sequences A or C' as described above and the probe preferably binds to a region B located between the ends of the binding sequences A and C' or to the complement thereof.

Even when using at least one sequence out of the 3 sequence regions of the two primers and the probe which is not specific for the nucleic acid to be detected, the overall specificity of the detection method is retained. If one of the primer sequences is not specific for the nucleic acid to be detected but also binds to other nucleic acids, a specific nucleic acid amplification product cannot be formed on the other nucleic acid since the second primer binding sequence is absent. Unspecific nucleic acid amplification products are not detected on the other nucleic acid if the specific binding sequence for the probe is absent. If the second primer sequence is also not specific for the nucleic acid to be detected, then a specific nucleic acid amplification product can only be formed on the other nucleic acid if both primer binding sequences are in the same nucleic acid amplification unit. This nucleic acid amplification product is also not detected since the specific binding sequence for the probe is absent. If the probe sequence is not specific for the nucleic acid to be detected but both primers are specific, no nucleic acid amplification products of the other nucleic acid are formed. If, in addition to the probe sequence, one of the two primer sequences is also not specific for the nucleic acid to

be detected, again no specific nucleic acid amplification product of the other nucleic acid can be formed. Unspecific nucleic acid amplification products of the other nucleic acid that may be formed contain other sequences in the probe binding region and are therefore not detected. If all three binding sequences for the two primers and the probe are not specific for the nucleic acid to be detected, no nucleic acid amplification product is formed if at least one of the two primer sequences is not located in a nucleic acid amplification unit of the other nucleic acid. If the probe sequence is not located in the nucleic acid amplification unit of the two primer sequences for the other nucleic acid, a specific nucleic acid amplification product of the other nucleic acid can indeed be formed but not detected. The only case in which a specific nucleic acid amplification product of the other nucleic acid can be formed and detected, is when all three sequences are within a nucleic acid amplification region. However, this can be avoided by appropriate selection of the sequences of the nucleic acid amplification unit, e.g. by not also selecting the primer hybridization sites from the same locus of the same organism that is not to be detected.

Another method of making the primers and probes specifically unselective is to use degenerate bases within the sequence. For this it is expedient to select the region in which the hybridization of the target nucleic acid with the primer or with the probe is to take place such that there are relatively few differences between the target sequence and another sequence which is not the sequence to be detected (e.g. of another microorganism). The differences which remain can be largely compensated by using degenerate bases at

the differing base positions. Thus differences in the primers (A or G) can be compensated by incorporating the base P (6H, 8H-3,4-dihydro-pyrimido[9,5-C][1,2]oxazin-7-one, e.g. Nucleic Acids Research, vol. 17, 24, 1989, p. 10373-10383). The same applies to pyrimidines where the base K is used (Nucleorides & Nucleotides, 16 (7-9), 1507-1511 (1997)). An even stronger degeneration is possible by using inosine (US-A-5,585,477; US-A-5,691,134; US-A-5,578,467; J.Biol.Chem. 260, 5, 2605-2608, 1985; Nucl.Acids Res. 1995, 23, 13, 2499-2505) since inosine allows base pairing with all four bases.

A further method of using non-complementary bases is to replace A by D (diaminopurine) or/and to replace C by M (methylcytosine).

In a further embodiment the amplificates are produced using nucleotides, particularly preferably mononucleotides which are each complementary to A, G, C and/or T. The region B or B' of the nucleic acid to be detected preferably contains all 4 natural nucleobases.

A technical advantage of the method according to the invention is that in multiple determinations of a sample a high degree of agreement of the measured values is achieved.

In the following the two aspects of the present invention are described on the basis of a HCV test. The nucleic acid sequence of HCV is for example described in EP-B-0 318 216. A section of a HCV genome is shown in fig. 4 which forms the basis of the method shown as the example. The sequence of HGBV-B which corresponds to the HCV sequence and from which the sequences for the

primers and probes have been taken are also shown in fig. 4.

Surprisingly it is possible to detect HCV-RNA specifically and reproducibly in positive HCV plasma samples in which the HCV-RNA was not sequence-specifically prepurified but was used directly from lysed plasma samples that were concentrated by means of glass surfaces despite the short amplified sequence of the nucleic acid to be detected. HCV-negative plasma samples result in no signal. This is surprising since the HCV-RNA genome is very susceptible to fragmentation in plasma lysates. The primers and probes that were used also give no signal with for example HIV plasma samples, HBV serum samples, chlamydia samples from urine or human DNA samples from whole blood which have also been concentrated by means of glass surfaces.

The method according to the invention can be used to avoid one or several of the disadvantages described for the prior art or to realize one or several of the following advantages. PCR cycles can be very much shorter. The overall time for the detection method can thus be shortened. The sensitivity of the test can be increased since less competition/displacement between the short complementary strand of the amplicon and the detection probe can take place. The specificity of the test is increased since the relative proportion of the internal detector region is increased in relation to the total amplicon. The ability to differentiate between subtypes can be increased. The test background can be reduced since short amplicons have less potential for unspecific hybridization. Consequently the signal-noise ratio can be increased. The reproducibility of the results can be increased since smaller target regions on

RNA genomes are less sensitive to RNA degradation. The potential for forming secondary structures is reduced.

The invention is elucidated in more detail by the following examples:

General

All oligonucleotides used are linear and single-stranded.

Example 1

Detection of HCV from human blood

a) Sample preparation:

RNA was isolated from plasma using the following sample preparation protocol:

1. mix plasma (420 µl) with 80 µl proteinase K (25 mg/ml) and vortex for a few seconds
2. add 500 µl lysis buffer (incl. 1 µg carrier-RNA (polyA)/ml): 5.4 M guanidinium thiocyanate; 10 mM urea; 10 mM Tris-HCl; 20 % Triton X 100; pH 4.4
3. vortex and subsequently shake for 10 min at RT
4. add 500 µl isopropanol-MGP (6 mg magnetic glass particles in isopropanol)
5. vortex and subsequently shake for 20 min at RT
6. magnetically separate the MGPs
7. remove and discard the supernatant
8. add 750 µl wash buffer: 20 mM NaCl; 20 mM Tris-HCl pH 7,5; 70 % ethanol
9. resuspend the MGPs on a vortex mixer and again separate magnetically
10. repeat wash process 5-times overall

11. add 100 µl DEMC water for the elution
12. shake for 15 min at 80 °C
13. separate magnetically
14. use 10 µl of the eluate in the RT-PCR

b) Cloning and preparation of the RNA standard:

The wild-type standard "pHCV-wt" was firstly obtained by amplifying a section of the HCV genome using the primers KY80 (5'-gcagaaagcgtctagccatggcgt-3', SEQ.ID.NO.1) and KY78 (5'-ctcgcaagcaccctatcaggcagt-3', SEQ.ID.NO.2) and the amplicon was subsequently cloned into the vector pBluescript SK+ by means of a so-called blunt end cloning. The plasmid was isolated after growing the bacterial cells, it was linearized by restriction enzymatic digestion and the corresponding RNA fragment was obtained by in vitro transcription and purified.

The RNA was quantified by photometric measurement of the absorbence at 260 nm.

All the molecular biological methods described here can be taken from the relevant method books (e.g. Maniatis et al.; Ausubel et al.).

c) RT-PCR assay

The amplification was carried out analogously to the method described in EP-A-0 699 768. Further details for constructing probes for fluorogenically labelled probes for the TaqMan™ method are described in the product description for the instruments TaqMan™LS-50B and ABI Prism 7700 from Perkin Elmer. The experiments were

carried out using the following primer and probe sequences:

forward primer: selected from the sequence between positions 390 and 417,
reverse primer: selected from the sequence between positions 421 and 448,
probe: selected from the sequence between positions 408 and 440 all with reference to the HGBV-B sequence from sequence HG22304 obtainable from the EMBL data bank emvrl or from Proc. Natl. Acad. Sci USA 1995, 92, 3401-3405 and/or from J. Virol. 69: 5621-5630. The sequence shown in figure 4 corresponds to positions 390 to 448 of this sequence so that the primer and probe positions can be directly converted.

Preferred primer/probe combinations are hence as follows:
forward primer selected from one of the sequences: 390-406, 390-408, 391-406, 391-408, 392-406, and 392-408,
reverse primer selected from one of the sequences: 427-448, 427-447, 427-446, 428-448, 428-447, 428-446, 429-448 and 429-447,
probe selected from one of the sequences: 408-436, 408-435, 408-434, 408-433, 408-432, 408-431, 408-430, 408-429, 408-428, 409-436, 409-435, 409-434, 409-433, 409-432, 409-431, 409-430, 409-429, 409-428, 410-436, 410-435, 410-434, 410-433, 410-432, 410-431, 410-430, 410-429, and 410-428, or preferably

forward primer: sequence from 390-406, 390-408, 391-406, 391-408, 392-406, and 392-408,
reverse primer: selected from one of the sequences: 423-448, 423-447, 423-446, 423-445, 423-444,

probe: selected from one of the sequences: 409-433, 409-432, 409-431, 410-433, 410-432, 410-431, 410-430, 410-429, 410-428, 409-430, 409-429, 409-428, 408-433, 408-432, 408-431, 408-430, 408-429, and 408-428 or particularly preferably:

forward primer: sequence from 390-406, 391-406, and 392-406,
reverse primer: selected from one of the sequences: 423-448, 423-447, 423-446, 423-445, 423-444,
probe: selected from one of the sequences: 409-433, 409-432, 409-431, 410-433, 410-432, 410-431, 410-430, 410-429, 410-428, 409-430, 409-429, 409-428, 408-433, 408-432, 408-431, 408-430, 408-429, and 408-428.

Example 2:

The primers and probes shown in fig. 5 were also used according to example 1. In this case the last 3 sequences are shown as their complement (probes or reverse primers). The first sequence is the forward primer, the sequence of TaqMan probes are shown in the 2nd to 4th lines. Lines 5-8 show the amplicons.

Example 3:

Further possible primer combinations and a HCV genomic sequence that corresponds to the amplicon formed therefrom are shown in fig. 6 and 7. In this case the combinations CK12-2/CK23-1/CK42 and CK11-1/CK21-1 and CK22-1/CK39 are preferred. The HCV genomic sequence that is shown has proven to be particularly preferred within the sense of the invention for the construction of primers and probes. In the last line the relevant HGBV-B

sequence is in each case also shown. In the case shown the primers and probes contain nucleotides with targeted degeneration. However, this does not mean that the oligonucleotides without degeneration cannot be suitable for the amplification of HCV.

Claims

1. Method for the detection of a nucleic acid comprising the steps

   - producing a plurality of amplificates of a section of this nucleic acid with the aid of two primers, one of which can bind to a first binding sequence (A) of one strand of the nucleic acid and the other can bind to a second binding sequence (C') which is essentially complementary to a sequence C which is located in the 3' direction from A and does not overlap A, in the presence of a probe with a binding sequence D which can bind to the third sequence (B) located between the sequences A and C or to the complement (B') thereof, wherein this probe contains a reporter group and a quencher group, using a polymerase having 5' nuclease activity and

   - detecting the nucleic acid by measuring a signal which is caused by the release of the reporter group,

   wherein the amplificates formed with the aid of the primers have a length of less than 75 nucleotides.

2. Method as claimed in claim 1, wherein the binding sequence D of the probe does not overlap one of the binding sequences of the primers.

3. Method as claimed in one of the previous claims, wherein at least one of the binding sequences is not specific for the nucleic acid to be detected.

4. Method as claimed in one of the previous claims, wherein the total length of the amplificates formed with the aid of the primers have a length of less than 61 nucleotides.

5. Method as claimed in one of the previous claims, wherein the probe is labelled with a fluorescence quencher as well as with a fluorescent dye.

6. Method as claimed in one of the previous claims, wherein at least one of the primers is not specific for the nucleic acid to be detected.

7. Method as claimed in claim 6, wherein two of the primers are not specific for the nucleic acid to be detected.

8. Method as claimed in one of the claims 6 and 7, wherein the probe is not specific for the nucleic acid to be detected.

9. Method as claimed in one of the previous claims, wherein nucleotides which are each complementary to A, G, C and T are used in the amplification.

Abstract

The invention concerns a method for the detection of a nucleic acid comprising the steps production of a plurality of amplificates of a section of this nucleic acid having a length of less than 100 nucleotides with the aid of two primers, one of which can bind to a first binding sequence (A) of a strand of the nucleic acid of the nucleic acid and the other can bind to a second binding sequence (C') which is essentially complementary to a sequence (C) which is located in the 3' direction from (A) and does not overlap (A), which can bind in the presence of a probe with a binding sequence (D) which can bind to a third sequence (B) which is located between the sequences A and C or to the complement (B') thereof, wherein this probe contains a reporter group and a quencher group using a polymerase having 5' nuclease activity and detecting the nucleic acid by measuring a signal which is caused by the release of the reporter group.

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:
    (i) Opposing an argument of unpatentability relied on by the Office, or
    (ii) Asserting an argument of patentability.

Fig. 1

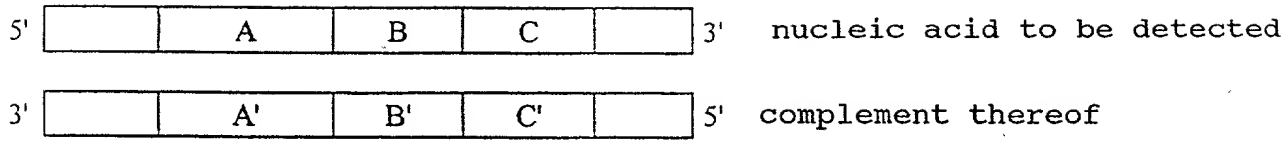
5'
A
B
C
3'
nucleic acid to be detected
3'
A'
B'
C'
5'
complement thereof

Fig. 2

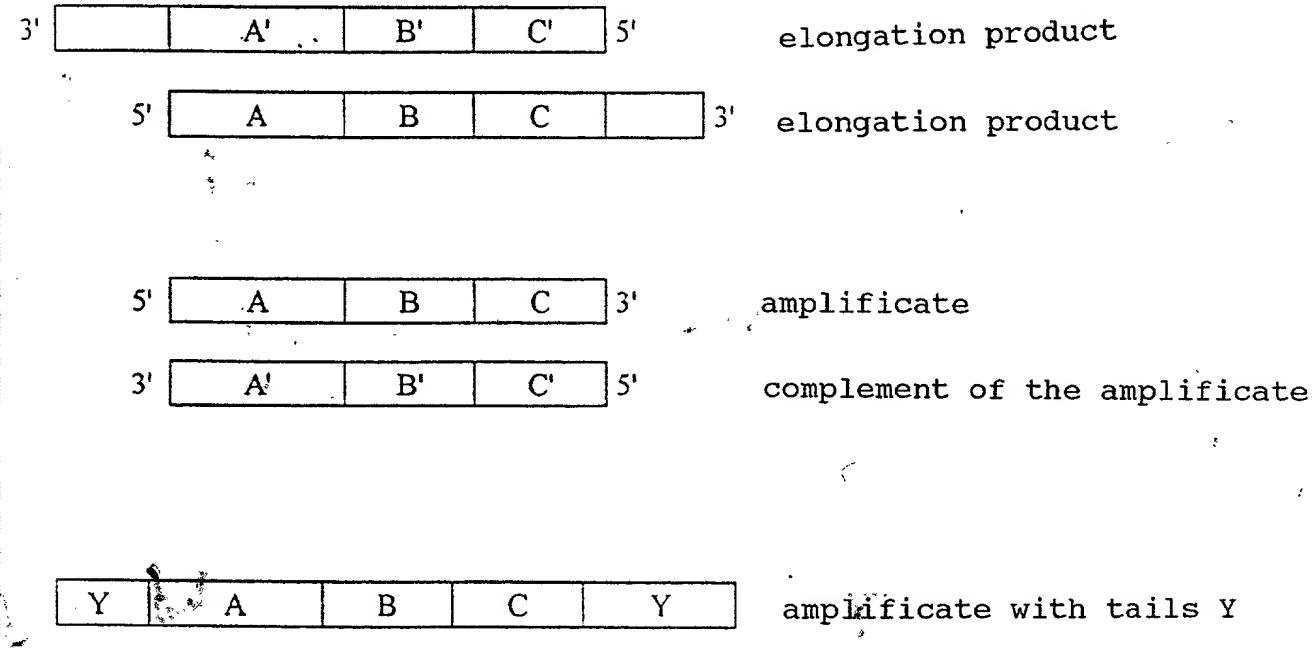
3'
A'
B'
C'
5'
elongation product
5'
A
B
C
3'
elongation product
5'
A
B
C
3'
amplificate
3'
A'
B'
C'
5'
complement of the amplificate
Y
A
B
C
Y
amplificate with tails Y

Fig. 3

530747

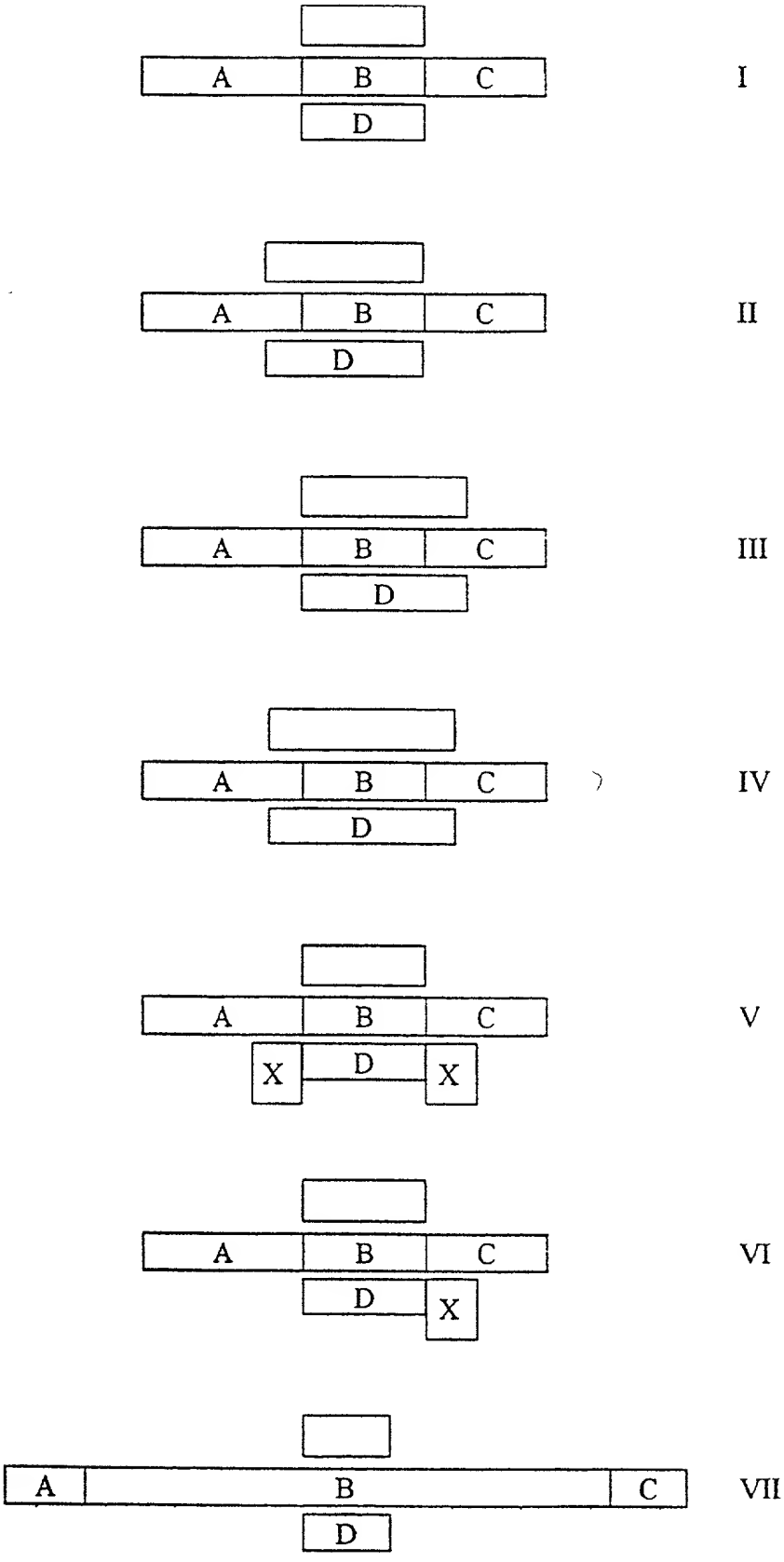

Fig. 4

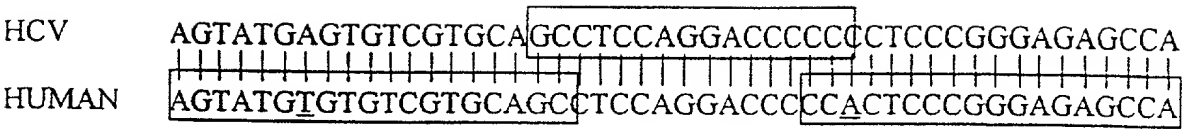
HCV
AGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCA
HUMAN
AGTATGTGTGTCGTGCAGCCTCCAGGACCCCCACTCCCGGGAGAGCCA

FIG. 5

```
HCVMCR01          AGTATGTGTGTCGTGCAGCC
MPF1                        CCAGGACCCCCCACTCCCGG
MPF1+1                    TCCAGGACCCCCCACTCCCGG
MPF2                        CCAGGACCCCCCACTCC
HCV_1A            AGTATGAGTGTCGTGCAGCCTCCAGGCCCCCCCCTCCCGGGAGAGCCA
HCV_1B            AGTATGAGTGTCGTGCAGCCTCCAGGCCCCCCCCTCCCGGGAGAGCCA
HCV_2B            AGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCCTCCCGGGAGAGCCA
HCV__MCR          AGTATGTGTGTCGTGCAGCCTCCAGGACCCCCCACTCCCGGGAGAGCCA
MPR1_rev&compl                GTGTGTCGTGCAGCCTCCAGGA
MPR2_rev&compl                  TCGTGCAGCCTCCAGGA
HCVMCR02_rev&compl                                CCACTCCCGGGAGAGCCA
      #1
                  ----------.----------.----------.----------.----------
```

FIG 6

HCV 261 5´-GGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACCATGA-3´ 333

Primer pair	Forward primer	Reverse primer
Foreward primer CK10/Reverse primer CK20	5´-CGTACTGCCTGATAGGGTGCT-3´	3´-CAGAGMATMTGGMATCGTGTAMG-5´
Foreward primer CK11/Reverse primer CK20	5´-CGTACTGCCTGATAGGGTGC-3´	3´-CAGAGMATMTGGMATMGTGTAMG-5´
Foreward primer CK10-1/Reverse primer CK20-1	5´-CGTACTGCCTIATAGGGTICT-3´	3´-CDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK11-1/Reverse primer CK20-1	5´-CGTACTGCCTIATAGGGTIC-3´	3´-CDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK10-2/Reverse primer CK20-2	5´-CGTAMTGMMTIATAGGGTIMT-3´	3´-MDGDIMDTMTGGMAPPKPGTAMG-5´
Foreward primer CK11-2/Reverse primer CK20-2	5´-CGTAMTGMMTIATAGGGTIM-3´	3´-MDGDIMDTMTGGMAPPKPGTAMG-5´
Foreward primer CK10/Reverse primer CK21	5´-CGTACTGCCTGATAGGGTGCT 3´	3´-CTTCAGAGCATCTGGCATCGTGTACG-5´
Foreward primer CK10-1/Reverse primer CK21-1	5´-CGTAMTGMMTIATAGGGTICT 3´	3´-CTTMDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK11-1/Reverse primer CK21-1	5´-CGTAMTGMMTIATAGGGTIC 3´	3´-CTTMDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK10-1/Reverse primer CK21-2	5´-CGTAMTGMMTIATAGGGTICT 3´	3´-CTPMDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK11-1/Reverse primer CK21-2	5´-CGTAMTGMMTIATAGGGTIC 3´	3´-CTPMDGDIMDTMTGGMATMGTGTAMG-5´
Foreward primer CK10-2/Reverse primer CK21-3	5 -CGTAMTGMMTIATAGGGTIMT 3´	3´-MTPMDGDIMDTMTGGMAPPKPGTAMG-5´
Foreward primer CK11-2/Reverse primer CK21-3	5 -CGTAMTGMMTIATAGGGTIM 3´	3´-MTPMDGDIMDTMTGGMAPPKPGTAMG-5´

HGBV-B 389 5´-CGTACTGCCTGATAGGGTCCTTGCGAGGGGATCTGGGAGTCTCGTAGACCGTAGCACATGC-3´ 449

FIG 7

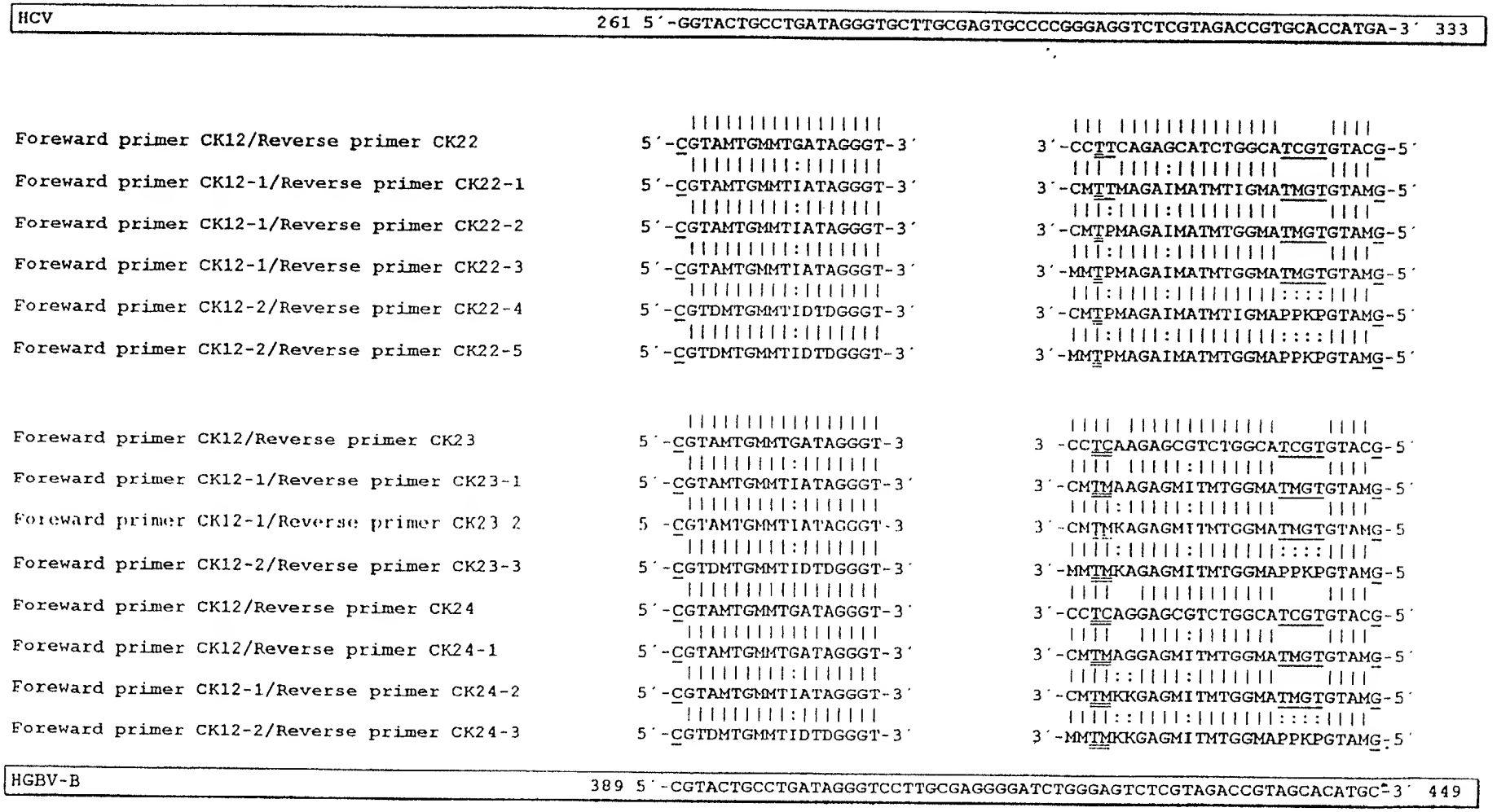
HCV
261 5′-GGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACCATGA-3′ 333
Foreward primer CK12/Reverse primer CK22
5′-CGTAMTGMMTGATAGGGT-3′
3′-CCTTCAGAGCATCTGGCATCGTGTACG-5′
Foreward primer CK12-1/Reverse primer CK22-1
5′-CGTAMTGMMTIATAGGGT-3′
3′-CMTTMAGAIMATMTIGMATMGTGTAMG-5′
Foreward primer CK12-1/Reverse primer CK22-2
5′-CGTAMTGMMTIATAGGGT-3′
3′-CMTPMAGAIMATMTGGMATMGTGTAMG-5′
Foreward primer CK12-1/Reverse primer CK22-3
5′-CGTAMTGMMTIATAGGGT-3′
3′-MMTPMAGAIMATMTGGMATMGTGTAMG-5′
Foreward primer CK12-2/Reverse primer CK22-4
5′-CGTDMTGMMTIDTDGGGT-3′
3′-CMTPMAGAIMATMTIGMAPPKPGTAMG-5′
Foreward primer CK12-2/Reverse primer CK22-5
5′-CGTDMTGMMTIDTDGGGT-3′
3′-MMTPMAGAIMATMTGGMAPPKPGTAMG-5′
Foreward primer CK12/Reverse primer CK23
5′-CGTAMTGMMTGATAGGGT-3
3 -CCTCAAGAGCGTCTGGCATCGTGTACG-5′
Foreward primer CK12-1/Reverse primer CK23-1
5′-CGTAMTGMMTIATAGGGT-3′
3′-CMTMAAGAGMITMTGGMATMGTGTAMG-5′
Foreward primer CK12-1/Reverse primer CK23 2
5 -CGTAMTGMMTIATAGGGT-3
3′-CMTMKAGAGMITMTGGMATMGTGTAMG-5
Foreward primer CK12-2/Reverse primer CK23-3
5′-CGTDMTGMMTIDTDGGGT-3′
3′-MMTMKAGAGMITMTGGMAPPKPGTAMG-5
Foreward primer CK12/Reverse primer CK24
5′-CGTAMTGMMTGATAGGGT-3′
3′-CCTCAGGAGCGTCTGGCATCGTGTACG-5′
Foreward primer CK12/Reverse primer CK24-1
5′-CGTAMTGMMTGATAGGGT-3′
3′-CMTMAGGAGMITMTGGMATMGTGTAMG-5′
Foreward primer CK12-1/Reverse primer CK24-2
5′-CGTAMTGMMTIATAGGGT-3′
3′-CMTMKKGAGMITMTGGMATMGTGTAMG-5′
Foreward primer CK12-2/Reverse primer CK24-3
5′-CGTDMTGMMTIDTDGGGT-3′
3′-MMTMKKGAGMITMTGGMAPPKPGTAMG-5′
HGBV-B
389 5′-CGTACTGCCTGATAGGGTCCTTGCGAGGGGATCTGGGAGTCTCGTAGACCGTAGCACATGC-3′ 449

FIG. 8

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HCV      267 5'CCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACCATGAGCACGAAT3' 341
             || |  ||||||||||||||||| |||||||| |  || ||||||||||||||||     ||||       | |
HGBV-B   383 5'CCATACCGTACTGCCTGATAGGGTCCTTGCGAGGGGATCTGGGAGTCTCGTAGACCGTAGCACATGCCTGTTATT3' 457
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Application Serial No: 09/530,747
Filed on May 4, 2000
Attorney Docket No. 4817/0R

Declaration and Power of Attorney for Patent Application

As the below named inventor(s), We hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SPECIFIC AND SENSITIVE NUCLEIC ACID DETECTION METHOD

the specification of which (check one)

[ ] is attached hereto.

[ ] was filed on _____ as

Application Serial No. _____

and was amended on _____ (if applicable).

[X] was filed on November 3, 1998 as

PCT International Application Serial No. PCT/EP98/06961

and was amended under PCT Article 19 on November 19, 1999 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			**Priority Claimed**	
197 48 690.8 (Number)	DE (Country)	4 November 1997 (Day/Month/Year Filed)	[X] Yes	[ ] No
198 14 001.0 (Number)	DE (Country)	28 March 1998 (Day/Month/Year Filed)	[X] Yes	[ ] No

198 14 828.3	DE	2 April 1998	[X]	[ ]
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application No.)	(Filing Date)	(Application No.)	(Filing Date)
(Application No.)	(Filing Date)	(Application No.)	(Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

PCT/EP98/06961	November 3, 1998	Pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the practitioners at Customer Number 22829 to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Fourth Inventor's Signature — Date

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